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Embryonic Chick Retinal Basal Lamina

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To study the biology of basal laminae in the developing nervous system the protein composition of the embryonic retinal basal lamina was investigated, the site of synthesis of its proteins in the eye was determined, and basal lamina assembly was studied *in vivo* in two assay systems. Laminin, nidogen, agrin, collagen IV, and XVIII are major constituents of the retinal basal lamina. However, only agrin is synthesized by the retina, whereas the other matrix constituents originate from cells of the ciliary body, the lens, or the optic disc. The synthesis from extraretinal tissues infers that the retinal basal lamina proteins must be shed from their tissues of origin into the vitreous body and from there bind to receptor proteins provided by the retinal neuroepithelium. The fact that all proteins typical for the retinal basal lamina are abundant in the vitreous body and a new basal lamina is only formed when the vitreous body was directly adjacent to the retina is consistent with the contention of the vitreous body having a function in retinal basal lamina formation. Basal lamina assembly was also studied after disrupting the retinal basal lamina by intraocular injection of collagenase. The basal lamina regenerated after chasing the collagenase with Matrigel, which served as a collagenase inhibitor. The basal lamina was reconstituted within 6 h. However, the regenerated basal lamina was located deeper in the retina than normal by reconstituting along the retracted neuroepithelial endfeet demonstrating that these endfeet are the preferred site of basal lamina assembly.

Key Words: **extracellular matrix; basal lamina; chick embryo; vitreous body; retina.**

INTRODUCTION

Basal laminae are approximately 50-nm-thick extracellular matrix sheets that are composed of members of the laminin family, collagen IV and XVIII, nidogen, agrin, and perlecan (for reviews see Yurchenko and Schitny, 1990; Timpl, 1996). Basal laminae form a physical barrier between epithelia and the underlying connective tissue, serve as a filter between mesangial cells and endothelial cells in the kidney, provide the ensheathment of muscle fibers and blood vessels, and contribute to the scaffolding of the alveoli of the lung. In the peripheral nervous system, basal laminae cover the surface of Schwann cells and are important in the formation of the neuromuscular junctions. The central nervous system has three types of basal laminae, one that outlines the vasculature and most likely contributes to the blood–brain barrier. The second type, the basal lamina of the pia, provides a cover of the outer surface of brain and spinal cord and separates the nervous tissues from the surrounding connective tissue. Yet another basal lamina in the central nervous system is the basal lamina of the neural retina, also referred to as the inner limiting membrane. The retinal basal lamina separates the retinal neuroepithelium from the vitreous body and probably has a similar functions as the pial basal lamina. Recent *in vivo* experiments have shown that the retinal and pial basal laminae are particularly important during the development of the nervous system by organizing and maintaining the cytoarchitecture of the pial and vitreal surface of the brain and retinal neuroepithelium, thereby having a crucial function during axonal navigation and histogenesis (Halfter, 1998; Halfter and Schurer, 1998).

The biochemical composition, the structure, and the assembly of basal laminae have mainly been studied using yolk sac tumor cells. Particularly the transplantable EHS mouse tumor (Orkin *et al.*, 1977), which produces large

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quantities of a basal lamina-like extracellular matrix, served as a source for the successful isolation and identification of most presently known basal lamina proteins (Kleinman *et al.*, 1982; Paulsson, 1992). While the composition of the EHS basal lamina is well studied, far fewer data are available on the composition, synthesis, and assembly of *in vivo* derived basal laminae and almost no data are available on the retinal and pial basal laminae. Since the retinal and pial basal laminae has been shown to be important in the development of central nervous system, we decided to take a closer look at the composition and synthesis of this extracellular matrix structure. The retinal basal lamina as an experimental system provides a number of advantages over basal laminae from other tissues, as the retinal basal lamina is not connected to a connective tissue layer and can therefore be isolated free of stromal proteins (Halfter and von Boxberg, 1992). Further, the chick eye has no vasculature in the retina and the inner limiting membrane is the only basal lamina of the chick retina focussing the study to a single type of basal lamina. Finally, the absence of endothelial cells in the retina and vitreous of the chick embryo allows a straightforward identification of retinal versus extraretinal tissues as the sites of synthesis of retinal basal lamina proteins in the eye.

EXPERIMENTAL PROCEDURES

Antibodies

The following monoclonal antibodies (MAbs) were used: MAb 3H11 to laminin (Halfter, 1993), MAb 6C4 to collagen XVIII (Halfter *et al.*, 1998), MAb 6D2 to agrin (Halfter, 1993), and MAb 33-2 to perlecan (Bayne *et al.*, 1984; obtained from the Developmental Studies Hybridoma Bank, Johns Hopkins University, Baltimore, MD). A rabbit antiserum to EHS mouse tumor laminin was purchased from Life Sciences (Gaithersburg, MD), and rabbit antisera to chick collagen IV, mouse perlecan, and mouse collagen IV were kindly provided by Drs. K. von der Mark (University Erlangen, Germany; Mayne *et al.*, 1982), R. Timpl (MPI for Biochemistry, Munich, Germany), and H. Kleinman (NIH, Bethesda, MD).

Gel Electrophoresis and Western Blotting

Retinal basal laminae were isolated from E10 chick retinae and solubilized in 8 M urea as described previously (Halfter and von Boxberg, 1992). Vitreous bodies were collected from E10 chick eyes, homogenized with a Polytron homogenizer, and centrifuged at 15,000 rpm for 20 min, and the supernatants were used as samples. Basal laminae and vitreous body samples were mixed with SDS sample buffer and loaded onto the gels. The proteins were separated by 3.6–14% SDS–PAGE and electrophoretically transferred to Immobilon P membrane filters (Millipore, Bedford, MA). After blocking with 5% skim milk in Ca, Mg-free Hanks' solution (CMF), the blots were incubated with the antibodies described above for 1 h. The blots were rinsed three times in CMF/milk, incubated with 1:5000 diluted (CMF/milk) alkaline phosphatase conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) for 1 h, and finally developed with NBT/BCIP.

Histology

Heads of E2.5 to E10 chick embryos were fixed in 4% paraformaldehyde in 0.1 M potassium phosphate buffer pH 7.4 for 1 h. After washing in CMF and cryoprotecting with 30% sucrose for 4 h, the specimens were embedded in OCT compound (Miles, Elkhart, IN) and sectioned in a horizontal plane with a cryostat at 10 μm. Sections were mounted on Superfrost slides (Fisher Scientific, Pittsburgh, PA) and incubated with hybridoma supernatants for 1 h. After three rinses, the sections were incubated with 1:500 Cy3-labeled goat anti-mouse antibody (Jackson ImmunoResearch) for an additional 1 h. After two final rinses, the specimens were examined with an epifluorescence microscope (Zeiss, Thornwood, NY). Basal lamina whole mounts from E6 chick retinae were prepared on polylysine-coated dishes or electron microscopy grids as described (Halfter and von Boxberg, 1992). The whole mounts were incubated in 2% Triton X-100 for 30 min, followed by 50 μg/ml of DNase I (Sigma) to remove all cellular component and stained with the various antibodies (see above). For transmission electron microscopy, the whole mounts were negatively stained with 2% uranyl acetate for 1 min or positively stained with 1% tannic acid followed by 1% OsO_4 and 2% uranyl acetate (Halfter and von Boxberg, 1992).

To visualize individual basal lamina proteins by electron microscopy, isolated retinal basal laminae were solubilized with 2 M guanidine hydrochloride and dialyzed against 0.1 M ammonium hydrogen carbonate buffer, pH 7.4 (Halfter and von Boxberg, 1992), and the extracts were sprayed onto freshly cleaved mica. Following rotary shadowing with Pt/carbon, the floated metal films were examined by TEM.

In Situ Hybridization

cDNA probes to collagen XVIII and agrin have been described previously (Halfter *et al.*, 1998; Tsen *et al.*, 1995). The cDNAs for nidogen and α1 collagen IV were obtained by antibody screening of a chick yolk sac library (Stratagene, La Jolla, CA) using the MAb 1G12 to nidogen and a polyclonal antiserum to chick collagen IV (Mayne *et al.*, 1982; see above). The 3.6-kb cDNA clone for nidogen has a nucleotide sequence that includes 1.4 kb of the coding region and the entire 2.2 kb of the 3′ untranslated region. The chick nidogen sequence is 70% identical to the nucleotide sequence of mouse nidogen cDNA. The 5.2-kb α1 collagen IV clone comprises almost the entire coding sequence and 519 bp of the 3′ untranslated region including a poly(A) tail. Its 5′ end begins 295 nucleotides short of the start codon. The sequence is 66% identical to the mouse α1 collagen IV cDNA sequence. Both sequences have been submitted to the Gene Bank (AF 239837 and 239838). The 1.074-kb cDNA clone for perlecan is from the 3′ end of the chick perlecan-coding region. It includes the complete sequence of the second EGF-like repeat, the third laminin G domain, and the stop codon. The sequence corresponds to domain 5 of the human and mouse perlecan and is about 60% identical to the mouse sequence (Osanger and Schneider, manuscript in preparation). The plasmids were linearized, and digoxigenin-labeled antisense and sense cRNA probes were synthesized from the templates using an RNA polymerase labeling kit (Boehringer Mannheim). The *in situ* hybridization procedure of tissue sections followed the procedure described by Schaeren-Wiemers and Gerfin-Moser (1993).

Transplantation

E3 chick eyes were cultured in DMEM/20% fetal calf serum. The grafting of tissues into these cultured eyes has been described previously (Halfter, 1996). Thirty hours after incubation, the eyes were fixed in 4% paraformaldehyde, 0.01% picric acid in 0.1 M phosphate buffer for 1 h, cryoprotected in 25% sucrose, and sectioned at 25 μm. Sections were immunostained as described above. Mouse transplants were identified by staining with a mouse-specific antibody to M6 (Lagenauer *et al.*, 1992; kindly provided by Dr. C. Lagenaur, University of Pittsburgh), whereas the heterochronic E8 chick transplants were identified by using an antibody to tenascin (Chiquet and Fambrough, 1984; Developmental Studies Hybridoma Bank). Some eyes were fixed in 2.5% glutaraldehyde in CMF and processed for transmission electron microscopy according to standard procedures.

Disruption and Regeneration of the Retinal Basal Lamina in Vivo

The retinal basal laminae were enzymatically removed *in vivo* by injecting 0.2–0.5μl of 100 U/ml (approximately 70 μg/ml) collagenase (Type VII, Sigma, St. Louis, MO) into E3 to E5 chick eyes (Halfter, 1998; Halfter and Schurer, 1998). As controls, 70 to 400 μg/ml chlostripain (Sigma) or dispase (Boehringer Mannheim) was injected as well.

For coinjection experiments, 80 μl of 100U/ml collagenase was mixed with 20 μl Matrigel (Kleinman *et al.*, 1986; Becton-Dickinson, Bedford, MA), and 0.5 to 1 μl of the mixture was injected into the chick eyes.

For basal lamina regeneration experiments, 0.5 to 1μl of 100 U/ml collagenase was injected into E3–E5 eyes, and the collagenase was chased at various time points by an injection of Matrigel. The Matrigel was diluted 1:4 with CMF (final protein concentration 1–1.2 mg/ml), and 1 μl of the diluted Matrigel was injected. The embryos were killed 3 h to 2 days after the Matrigel injections, and sections through the heads were stained with antibodies to laminin, nidogen, collagen XVIII, collagen IV, and agrin to determine the presence of an intact basal lamina in the eyes. Successful basal lamina regeneration *in vivo* was obtained with five different batches of Matrigel, one of which was "growth factor reduced." For controls, Matrigel was replaced with fetal bovine serum, laminin 1 from EHS mouse tumor, collagen IV (each from Life Sciences), and collagen I isolated from rat tail tendons. The final concentration of each of these proteins was between 1 and 1.5 mg/ml. The protein concentrations were determined as described by Minamide and Bamburg (1990). To visualize individual neuroepithelial cells and the basal lamina at the same time, the retinae were stained by mounting them, scleral side down, onto DiI-coated membrane filters as described previously (Halfter, 1998). Following immunolabeling with an anti-laminin MAb (see above) and an FITC-labeled secondary antibody, the retinae were removed from the filters, embedded in 1% agarose and sectioned with a vibratome at 250 μm.

Collagenase Assay

Collagenase activity was spectrophotometrically measured at 343 nm using as a substrate the synthetic peptide FALGPA (Sigma) at a final concentration of 0.125 mM (Van Wart and Steinbrink, 1981). Inhibition of collagenase activity by Matrigel, 1,10-phenantroline, and EDTA was measured by incubating 20 μl of collagenase at a concentration of 1000 U/ml with various concentrations of the inhibitors for 15 min and measuring the enzyme activity in a final reaction volume of 1 ml. Matrigel at concentrations ranging from 1 to 0.25 mg/ml showed a linear relationship of collagenase inhibition and Matrigel concentration. For example, 1 mg/ml Matrigel inhibited collagenase at 200 U/ml by approximately 40%. One millimolar of 1,10-phenantroline and 20 mM of EDTA inhibited 200 U/ml collagenase by 100 and 40%, respectively (van Wart and Steinbrink, 1981).

RESULTS

Constituents of the Retinal Basal Lamina

Immunocytochemical staining of cross-sections of paraformaldehyde-fixed eyes showed that major constituents of the retinal basal lamina are laminin (Fig. 1a), nidogen (Fig, 1b), agrin (Fig. 1c), and collagen XVIII (Fig. 1d). Unfixed basal lamina whole mount preparations (Halfter *et al.*, 1987) were also probed with these antibodies, and all of the proteins detected in tissue sections were also found in the whole mounts (Fig. 1f). Perlecan was detectable in cross-sections and in basal lamina whole mounts, though it was less abundant than agrin and collagen XVIII (not shown). Collagen IV was only detectable in the retinal basal lamina after a preincubation of the sections with 10 μg/ml pronase. The requirement for pronase treatment for the detection of collagen IV in the retinal basal lamina was unique as all other basal laminae were brightly stained without such pretreatment (Fig. 1e). Collagen IV was also detectable in whole mounts of unfixed retinal basal lamina (not shown), and its presence was further confirmed by Western blotting (see below) and by solubilizing purified retinal basal laminae and visualizing its protein components by rotary shadowing: collagen IV monomers and dimers in the typical appearance of a globular NC1 domain with extending long triple helical domains were among the most frequently seen proteins in these preparations (Fig. 1j). The basal lamina whole mounts were also probed by immunostaining with antibodies to NCAM (not shown) and by transmission (TEM, Figs. 1g and 1i) and scanning electron microscopy (not shown). All three preparation methods demonstrated that the isolated basal lamina whole mounts were clean sheets of extracellular matrix; free of plasma membranes, cellular debris, or organelles (Figs. 1g and 1i); and were comparable in their structure to intact basal laminae *in vivo* (Fig. 1h).

The presence of laminin, nidogen, agrin, and collagen IV and XVIII in retinal basal lamina was also shown by Western blotting using purified basal lamina preparations as samples (Halfter and von Boxberg, 1992). Laminin and nidogen appeared as bands of 220 (Fig. 2, lane 1) and 160 kDa (Fig. 2, lane 3), respectively, whereby the polyclonal antiserum to mouse laminin 1 recognized the 200-kDa β-chain but not the larger α-chain of the chick retinal laminin, consistent with earlier studies (Halfter *et al.*, 1987). Agrin and collagen XVIII, both heparan sulfate proteoglycans, appeared as diffuse smears of 600 and 300 kDa,

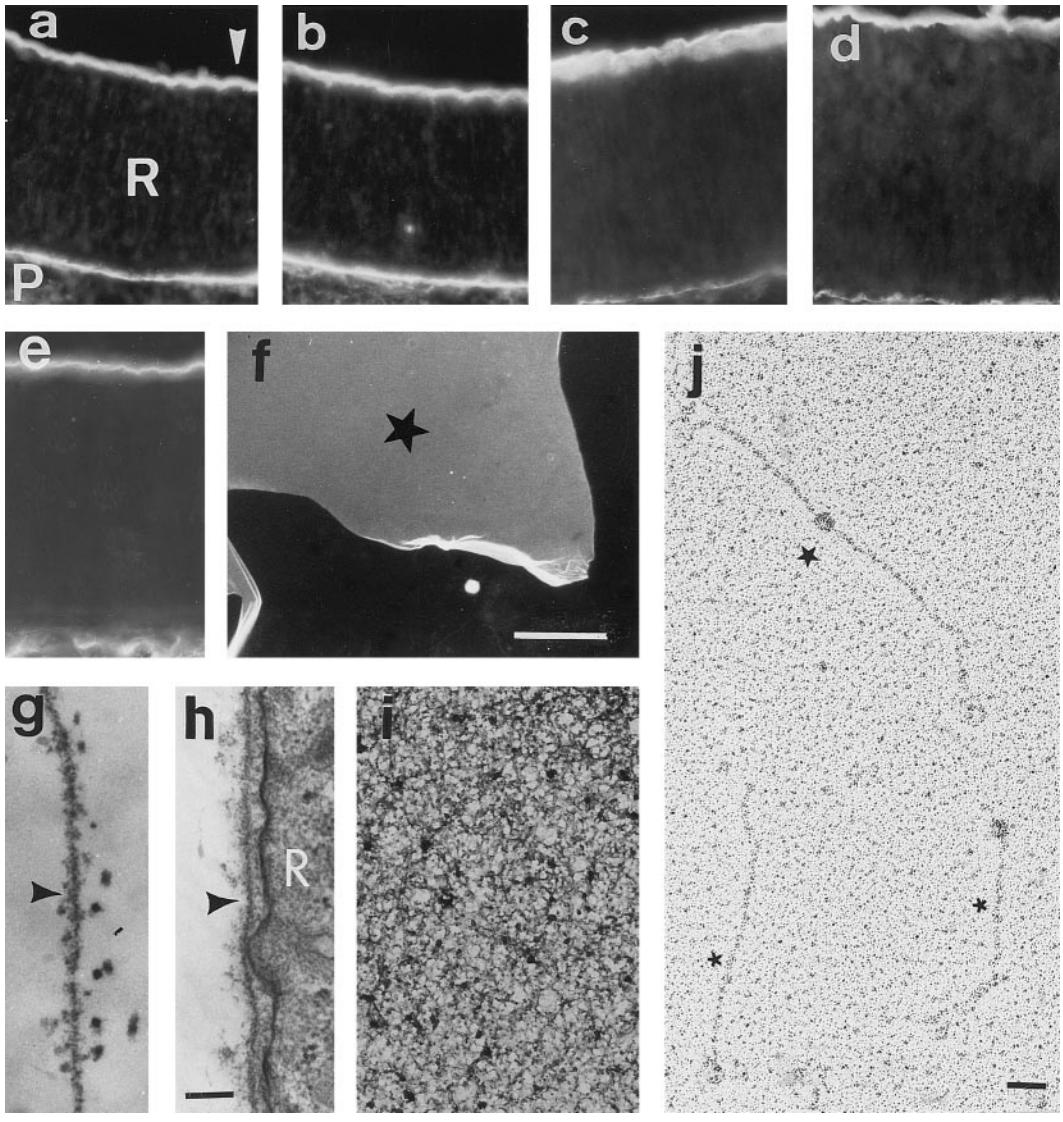


FIG. 1. Extracellular matrix constituents of the retinal basal lamina as shown by immunofluorescence micrographs of cross-sections through E6 chick retina (a–e). Strong staining of the retinal (R) basal lamina (arrowhead) was found by immunolabeling with antibodies to laminin (a), nidogen (b), agrin (c), collagen XVIII (d), and collagen IV (e). The proteins were also detected in Bruchs membrane, an extracellular matrix sheath of the pigment epithelial (P) that includes a basal lamina and adjacent connective tissue. Basal lamina whole mounts were also strongly labeled with these antibodies as exemplified by showing a basal lamina whole mount (star) stained with anti-laminin (f). The basal lamina whole mounts are clean sheets of extracellular matrix a shown by electron microscopy of cross-sections (g) and *en face* views (i). A cross-section of an intact basal lamina on the retina surface (R) is shown in (h) for comparison. Rotary shadowing of the proteins extracted from these basal laminae show numerous collagen IV dimers (large star) and monomers (small stars) and confirm the presence of collagen IV in the retinal basal lamina (j). Bar: a–f, 50 μm; g–j, 100 nm.

typical for proteoglycans (Fig. 2, lanes 5 and 7), and collagen IV appeared as a 180-kDa doublet (Fig. 2, lane 9). The immunostained bands around 300 and 900 kDa represent covalently linked oligomers of collagen IV. Western blotting showed that the proteins typical for the retinal basal lamina were also present in vitreous body. The banding pattern of laminin, nidogen, agrin, collagen IV, and collagen XVIII in vitreous body samples were similar to those from basal lamina samples (Fig. 2, lanes 2, 4, 6, 8, and 10).

To assure that extracellular matrix constituents from the vitreous body, such as collagen IX, tenascin, and fibronectin, did not contaminate the basal lamina preparations, we also stained the Western blots with an antibody to tenascin (Fig. 2, lanes 11 and 12) and collagen IX (not shown). As

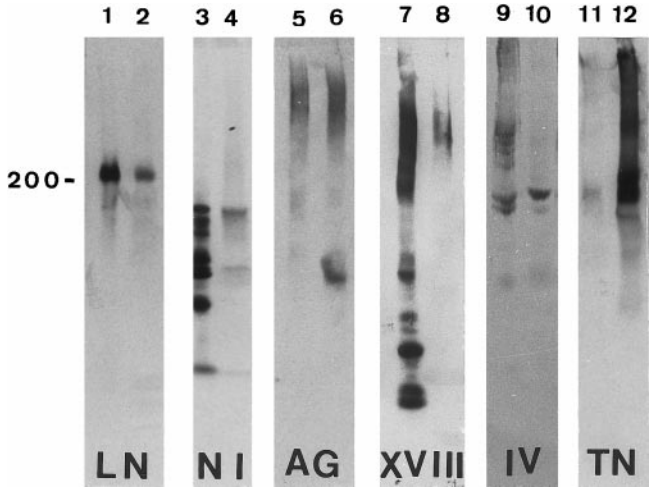


FIG. 2. Western blots showing extracellular matrix constituents of the retinal basal lamina (lanes 1, 3, 5, 7, 9, and 11) and vitreous body (lanes 2, 4, 6, 8, 10, and 12). Protein bands of laminin (LN, lanes 1 and 2), nidogen (NI, lanes 3 and 4), agrin (AG, lanes 5 and 6), collagen XVIII (XVIII, lanes 7 and 8), and collagen IV (IV, lanes 9 and 10) were found in basal lamina and vitreous body samples. The banding pattern of tenascin (TN) is shown in lanes 11 and 12 to demonstrate that the basal lamina samples (lane 11) were essentially free of vitreous body proteins (lane 12). Degradation bands were observed for nidogen and collagen XVIII from the basal lamina samples.

expected, abundant tenascin and collagen IX were found in vitreous body (Fig. 2, lane 12) but little tenascin and collagen IX in the basal lamina samples (Fig. 2, lane 11), confirming that our basal lamina preparations are essentially free of vitreous body components.

Origin of the Retinal Basal Lamina Proteins in the Developing Eye

To determine the site of synthesis of the retinal basal lamina proteins in the eye, we performed *in situ* hybridization studies on cross-sections of E5, E6, and E10 eyes using cRNA probes to nidogen, collagen XVIII, agrin, the α1-chain of collagen IV, and perlecan. E2.5 and E3 eyes were also processed for *in situ* hybridization studies of collagen XVIII, collagen IV, and agrin mRNAs. Collagen XVIII mRNA was detected in the future ciliary body and in the optic disc (Figs. 3a and 5a) and was undetectable in the neural retina at all stages of eye development studied (Figs. 3a and 5b). Similarly, nidogen mRNA was detected in the ciliary body and the optic disc (Figs. 3b and 5c), but not, or only in very minor quantities, in the neural retina (Fig. 5d). In contrast to collagen XVIII mRNA, nidogen mRNA was also abundantly expressed by the lens epithelial cells (Figs. 3b and 5c). Agrin mRNA was detected in ganglion cells of the retina and was the only basal lamina protein that was synthesized by the neural retina (Figs. 4a and 5f). According to the centroperipheral maturation of the retina, there were many more agrin-positive ganglion cells in the central than in the peripheral portion of an E5 retina, and the density of ganglion cells of the peripheral E5 retina resembled that of an E3 central retina. Agrin mRNA was not detectable in the ciliary body or the lens epithelium (Figs. 4a and 5e). The mRNAs of perlecan (Figs. 4b, 5g, and 5h) and the α1-chain of collagen IV (Figs. 4c, 5i, and 5j) were detected in the lens, but not in retina or the ciliary body. Minor quantities of perlecan mRNA were also found long blood vessels in the optic disc (not shown). Abundant α1 collagen IV mRNA was found in the optic disc, in the optic nerve, and in the periocular connective tissue (Figs. 5g, 5h, 12a, and 12b). At E2.5, the only site of collagen IV synthesis in the eye was the lens vesicle (not shown).

Formation of the Retinal Basal Lamina in Organ Cultured Eyes

To study the *de novo* formation of the retinal basal lamina *in situ* we used an organ culture system of E3 or E4 chick eyes (Halfter and Deiss, 1986). Prior to culture, small parts of the central retina were removed and replaced by grafts from (a) E4 chick neural retina, (b) E5 chick optic tectum, (c) E5 chick dorsal root ganglia, and (d) E5 chick periocular connective tissue. Earlier studies have shown that appropriate tissue grafts from chick and mouse embryos integrate into the host within 8 h with an almost perfect alignment and a continuous basal lamina between graft and host tissues (Halfter, 1996). To determine the origin of the newly synthesized basal lamina we grafted mouse retina into chick hosts and used chick-specific basal lamina antibodies to determine the origin of the basal lamina proteins on top of the transplants. Staining of sections through the cultured eyes with antibodies to laminin (Fig. 6a), nidogen, agrin, and collagen XVIII revealed in all experimental cases ($n = 10$) a continuous, host-derived basal lamina formed over the mouse retinal transplants (Fig. 6a). The basal lamina of the grafts was continuous with the basal lamina of the host retinae. A host-derived basal lamina was also established when the mouse (Fig. 6b, $n = 12$) or chick (Fig. 6f, $n = 10$) retinal grafts were flipped and implanted with the ventricular side facing the vitreous of the host eye. Since the ventricular side of the retina normally has no basal lamina, the basal lamina of the graft had to be synthesized *de novo*. TEM studies showed that these newly synthesized basal laminae on the surface of the inverted retinal transplants were ultrastructurally indistinguishable from a normal retinal basal lamina (compare Figs. 6g and 6h, $n = 5$). When chick optic tectum that was stripped of its pial basal lamina was implanted, a continuous basal lamina formed over the transplants (Fig. 6d, $n = 10$) showing that nonretinal neuroepithelium promotes *de novo* basal lamina formation as well. Transplants of dorsal root ganglia (Fig. 6e; $n = 10$) and periocular connective tissue (not shown, $n = 10$) were not covered with a basal lamina, showing that tissue from the periph-

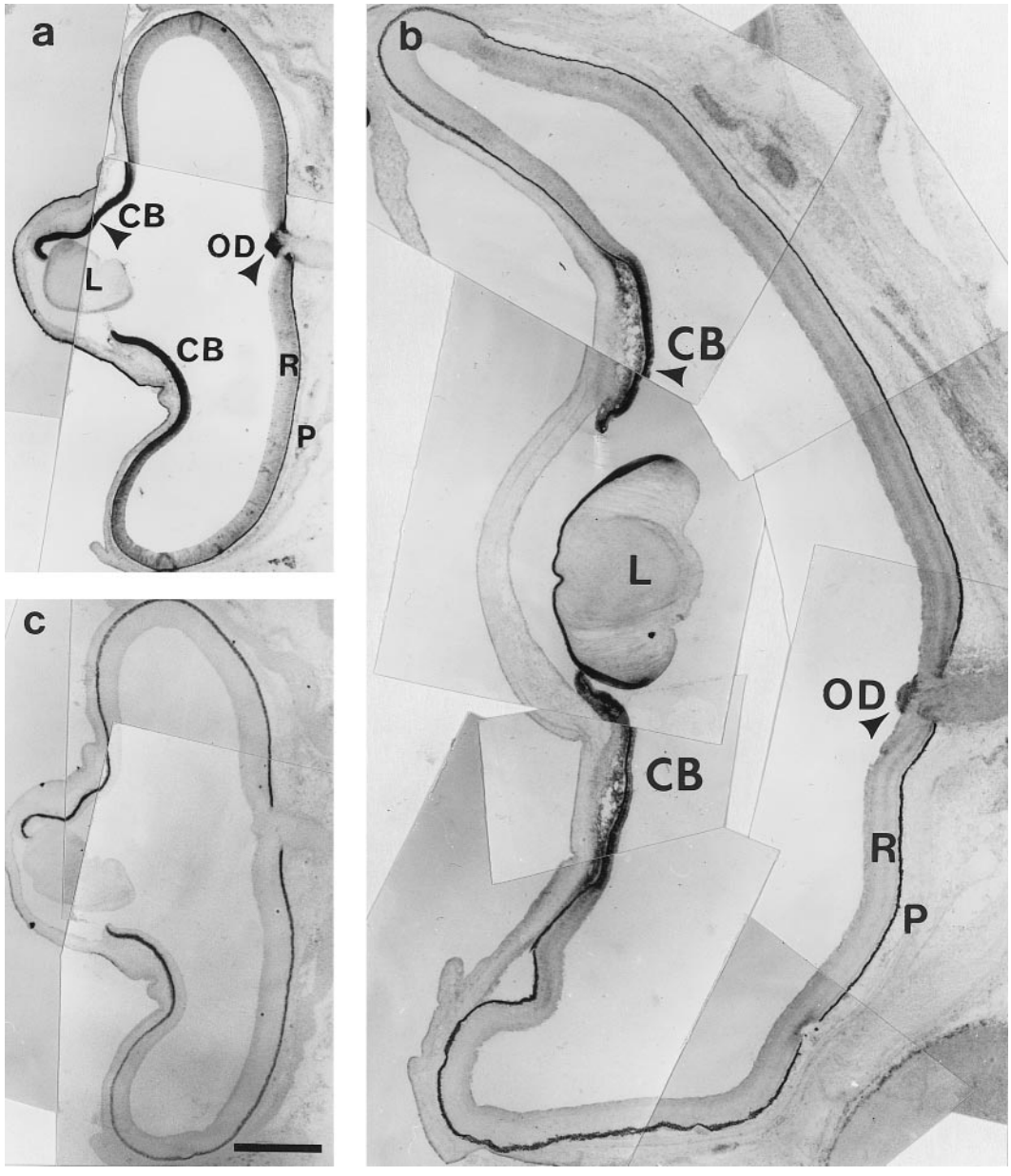


FIG. 3. Light micrographs of cross-sections through E5 (a, c) and E7 chick eyes (b) showing the distribution of collagen XVIII (a) and nidogen (b) mRNAs in the developing chick eye. As a control, the sense probe to collagen XVIII is shown for comparison in (c). Collagen XVIII and nidogen mRNAs are abundant in ciliary body (CB) and the optic disc (OD). P, pigment epithelium; R, retina. Nidogen mRNA is also detectable in the epithelial layer of the lens (L). Bar: 400 μm.

eral nervous system and connective tissue did not provide appropriate receptors for basal lamina formation in the eye.

Function of the Vitreous Body in Basal Lamina Formation

As shown previously in this article, most basal lamina proteins of the chick retina originate from the ciliary body, lens, and optic disc and are shed into the vitreous. To determine whether lens or vitreous body are essential for basal lamina formation we grafted retinal transplants with the scleral side facing the vitreous of the hosts. Further, the lens or lens and vitreous body were removed prior to culture. After 24 h of incubation, sections through the eyes were stained with antibodies to basal lamina proteins to reveal the presence or absence of a new basal lamina on top

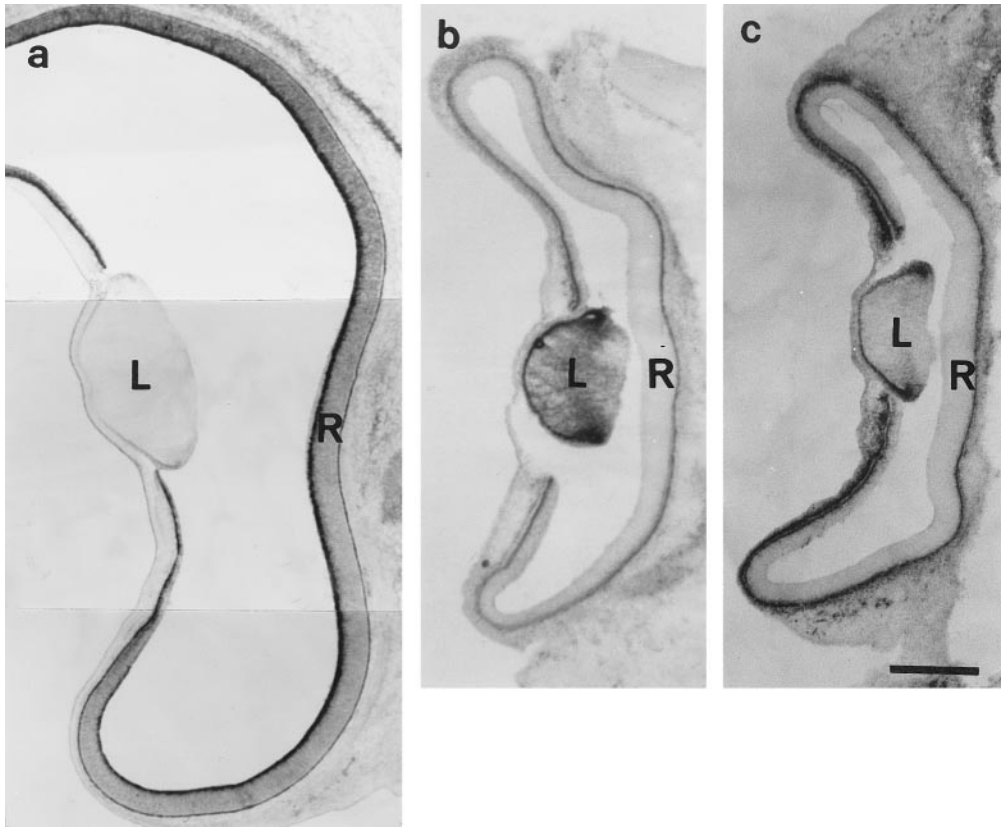


FIG. 4. Light micrographs of cross-sections through E6 (a) and E5 chick eyes (b, c) showing the distribution of agrin (a), perlecan (b), and α1 collagen IV (c) mRNAs in the developing chick eye. Agrin mRNA is abundant in the ganglion cell layer of the retina (R, a) whereas the mRNAs of perlecan (b) and α1 collagen IV (c) are abundant in the lens (L) and the periocular connective tissue. Bar, 400 μm.

of the inverted retinal grafts. The staining showed that in the absence of the lens a new basal lamina was formed in every case (Fig. 7a, $n = 12$). A basal lamina, however, did not form when the vitreous body had been removed (Fig. 7c, $n = 11$), showing that the vitreous body is essential for basal lamina formation in the neural retina.

Regeneration of the Retinal Basal Lamina in Vivo

As shown previously (Halfter, 1998), the retinal basal lamina can experimentally be removed *in vivo* by injecting bacterial collagenase into developing chick eyes. While injections of collagenase at a concentration of 100 U/ml (approximately 70 μg/ml) consistently led to the removal of the entire basal lamina of the neural retina ($n = 85$), injections of the nonspecific bacterial proteases chlostripain ($n = 6$) and dispase ($n = 15$) required at least 10 times higher concentrations to even cause a partial disruption to the retinal basal lamina, demonstrating that the removal of the retinal basal lamina by collagenase is due to the collagenolytic activity of the enzyme and not due to nonspecific proteolysis by potentially contaminating proteases.

Current experiments also showed that the disruption of the basal lamina was complete in less than 4 h after enzyme injection ($n = 32$), consistent with previous investigations (Halfter, 1998). The fact that the basal lamina does not regenerate even days after collagenase injection (Halfter, 1998) suggested to us that the injected collagenase continues to be active during further development and digests any new collagen molecules that are synthesized in the eye and that are required for the establishment of a new basal lamina. If this assumption was correct, we hoped that sequential injections of collagenase and a collagenase inhibitor should allow basal lamina regeneration by limiting the activity of the enzyme to a defined period of time. Using a spectrophotometrical enzyme assay and testing a variety of proteins and zinc chelators as potential collagenase inhibitors, we found that, in addition to the well-established collagenase inhibitors EDTA and 1,10-phenantroline, Matrigel, a basal lamina extract from the EHS mouse tumor, inhibited collagenase activity. In the *in vitro* assays, the inhibition of 1 mg/ml Matrigel was comparable to the inhibitory activity of 20 mM EDTA, but less efficient than 1 mM 1,10-phenantroline. Since Matrigel, unlike phenan-

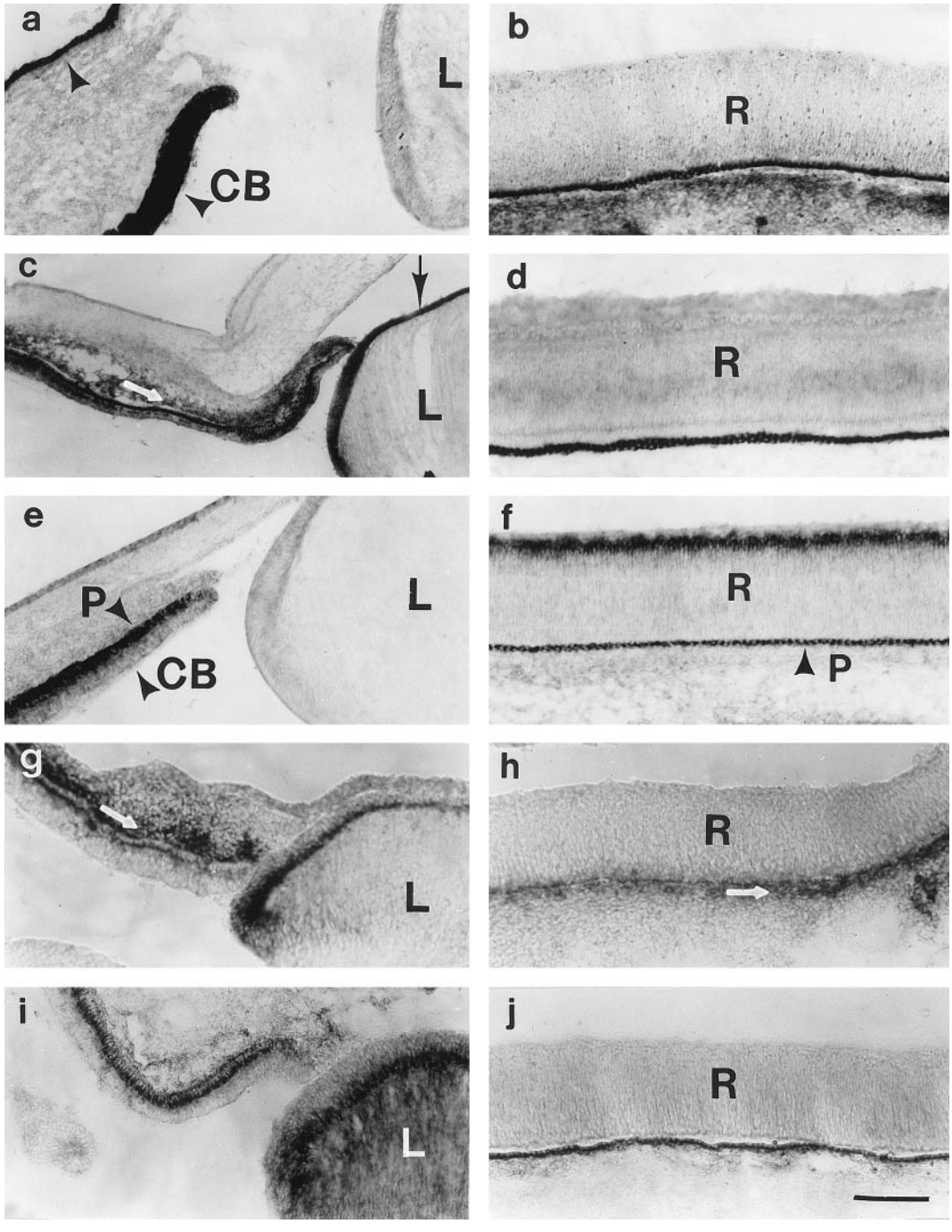


FIG. 5. High power micrographs showing *in situ* localization of mRNA for collagen XVIII (a, b), nidogen (c, d), agrin (e, f), perlecan (g, h), and α1 collagen IV (i, j) mRNAs in the ciliary margin and the lens (a, c, e, g, i) and the neural retina (b, d, f, h, j). Abundant message of collagen XVIII, and nidogen was found in the ciliary margin of the eye. In addition, nidogen mRNA was abundantly expressed by the lens epithelial cells. Agrin was found in the ganglion cell layer of the retina, but not in the lens and the ciliary margin. The cells responsible for agrin production in the retina are the ganglion cells (f). Perlecan and α1 collagen IV mRNAs were found in the lens (L) and the periocular connective tissue (arrows in g, h). P, pigment epithelium. Bar, 100 μm.

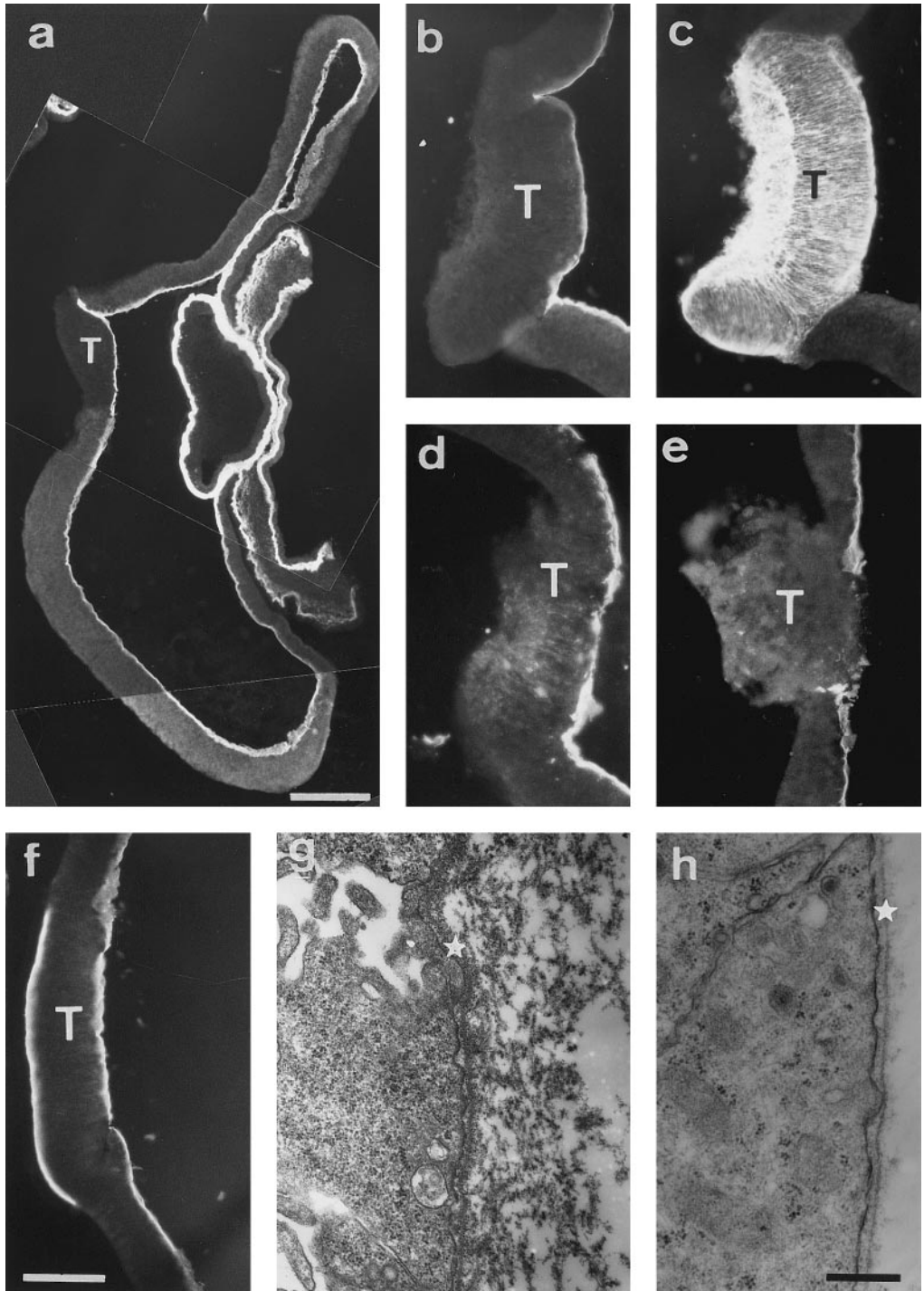


FIG. 6. Immunofluorescence micrographs of cross-sections through organ-cultured chick eyes showing the *de novo* synthesis of basal laminae after intraocular transplantation. E15 mouse retina (a–c), E6 chick retina (f–h), E6 chick optic tectum (d), and E6 dorsal root ganglia (e) transplants (T) were grafted into E3 chick eyes. The eyes were cultured for 1 day, and sections through these eyes were stained with a monoclonal antibody specific to chick laminin. A low power micrograph through an entire organ-cultured eye (a) shows the neat integration of the grafts into the host retina and demonstrates a continuous basal lamina of chick host and mouse graft. The mouse graft shown in (b) was transplanted with its scleral side facing the vitreous of the host to demonstrate the *de novo* synthesis of the basal lamina over the graft. The mouse origin of the transplant is shown by staining of the adjacent section with an antibody specific for mouse nervous tissue (c). A tectal graft (d) was also covered with a new basal lamina; however, a dorsal root ganglion transplant (e) was not. The newly synthesized basal laminae were morphologically indistinguishable from a normally grown retinal basal lamina as shown in (f) to (h). As shown by immunostaining for laminin, a new basal lamina has formed on the scleral side of an inverted chick retinal graft. The former basal lamina is still present at the outer surface of the transplant. Electron microscopy shows that these newly synthesized basal laminae (stars) are morphologically indistinguishable (g) to the basal lamina of a control retina (h). Bar: a, 200 μm; bf, 100 μm; g and h, 0.5 μm.

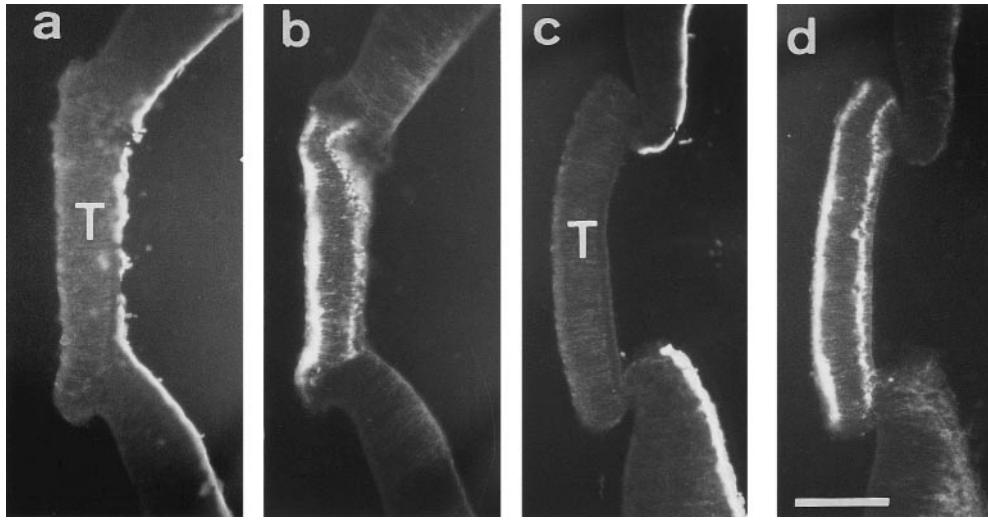


FIG. 7. Fluorescence micrographs of cross-sections through organ-cultured eyes showing the critical role of the vitreous body in the formation of the retinal basal lamina. The retinal transplants (T) were from E8 chick embryos and grafted with the scleral surface facing the vitreal surface of the E3 host. The eyes were cultured for 1 day. The eye in (a) and (b) was cultured after removing the lens. The eye in (c) and (d) was cultured after removing lens and vitreous body. Note that in the absence of the lens, the retinal basal lamina had formed (a), whereas in the absence of the vitreous body, a new basal lamina had not formed over the grafts (c). The identity of the grafts was confirmed by staining adjacent sections with antibodies to tenascin (b, d), which is present at the inner and outer plexiform layers of the E8 retinal grafts but not present in the E4 host. Bar, 100 μm.

troline and EDTA, is nontoxic and does not have any obvious side effect on eye development when intraocularly injected (not shown), we concentrated our further *in vivo* studies on Matrigel as a potential collagenase inhibitor.

That Matrigel could neutralize the collagenolytic activity of collagenase *in vivo* and thereby protect the retinal basal lamina from falling apart was shown by mixing and coinjecting collagenase and Matrigel at a ratio of 1 U collagenase to 10 μg Matrigel into chick eyes. Staining of cross-sections with antibodies to laminin, nidogen, collagen XVIII, and agrin showed that the retinal basal lamina remained intact in every coinjected eye (Fig. 8a, $n = 23$). Coinjections of collagenase with fetal bovine serum (Fig. 8c; $n = 9$), trypsin inhibitor ($n = 5$), laminin 1 ($n = 10$), collagen I (Figs. 8d and 8e; $n = 10$), or collagen IV ($n = 7$) at the same or higher protein concentrations than Matrigel consistently resulted in the complete loss of the retinal basal lamina showing that Matrigel uniquely protected the retinal basal lamina from the disruption by collagenase. To determine the minimum concentration of Matrigel necessary to protect the retinal basal lamina from collagenase-induced disruption, collagenase was mixed with decreasing concentrations of Matrigel, and the mixtures were injected into chick eyes. At a ratio of 1 U collagenase to 10 μg Matrigel (final concentration of Matrigel 1 mg/ml), the retinal basal laminae were rescued in all cases ($n = 10$). At a ratio of 1 U collagenase to 5 μg Matrigel (final concentration of Matrigel 0.5 mg/ml) the basal laminae of 50% of the injected eyes ($n = 6$) were rescued, and at a ratio of 1U to 2.5μg (final concentration of Matrigel 0.25 mg/ml), the basal lamina of one out of 11 injected eyes was rescued.

To find out whether sequential injections of collagenase and Matrigel leads to basal lamina regeneration, we injected collagenase into chick eyes and chased it, 8 h later, with Matrigel at a protein concentration of 1 mg/ml. The presence or absence of the retinal basal lamina was examined 24 h after the Matrigel injection by staining cross-sections of the eyes with antibodies to laminin, agrin, collagen XVIII, and nidogen. In all cases (Figs. 9 and 10d; $n = 66$), a new basal lamina had formed. The regenerated basal lamina was ectopically located underneath the optic fiber layer and was less regular than a normal basal lamina (Fig. 9), which allowed us to unequivocally distinguish it from a normal, previously undisrupted basal lamina (compare Figs. 9a and 9b, 10b and 10c, and 10d and 10e). The deeper than normal location of the basal lamina was due to the altered location of the neuroepithelial endfeet that serve as the basal lamina nucleation sites and that had retracted from the retinal surface after basal lamina disruption (Halfter, 1998; see below).

Since the monoclonal antibodies to detect the basal lamina proteins were chick specific and Matrigel is a mouse-derived extracellular matrix preparation, the new basal lamina had to be synthesized by the chick host and was not, or only to a minor extent, formed from the extracellular matrix proteins of the injected Matrigel. This was consistent with the fact that Matrigel-derived mouse collagen IV or perlecan were not incorporated into the

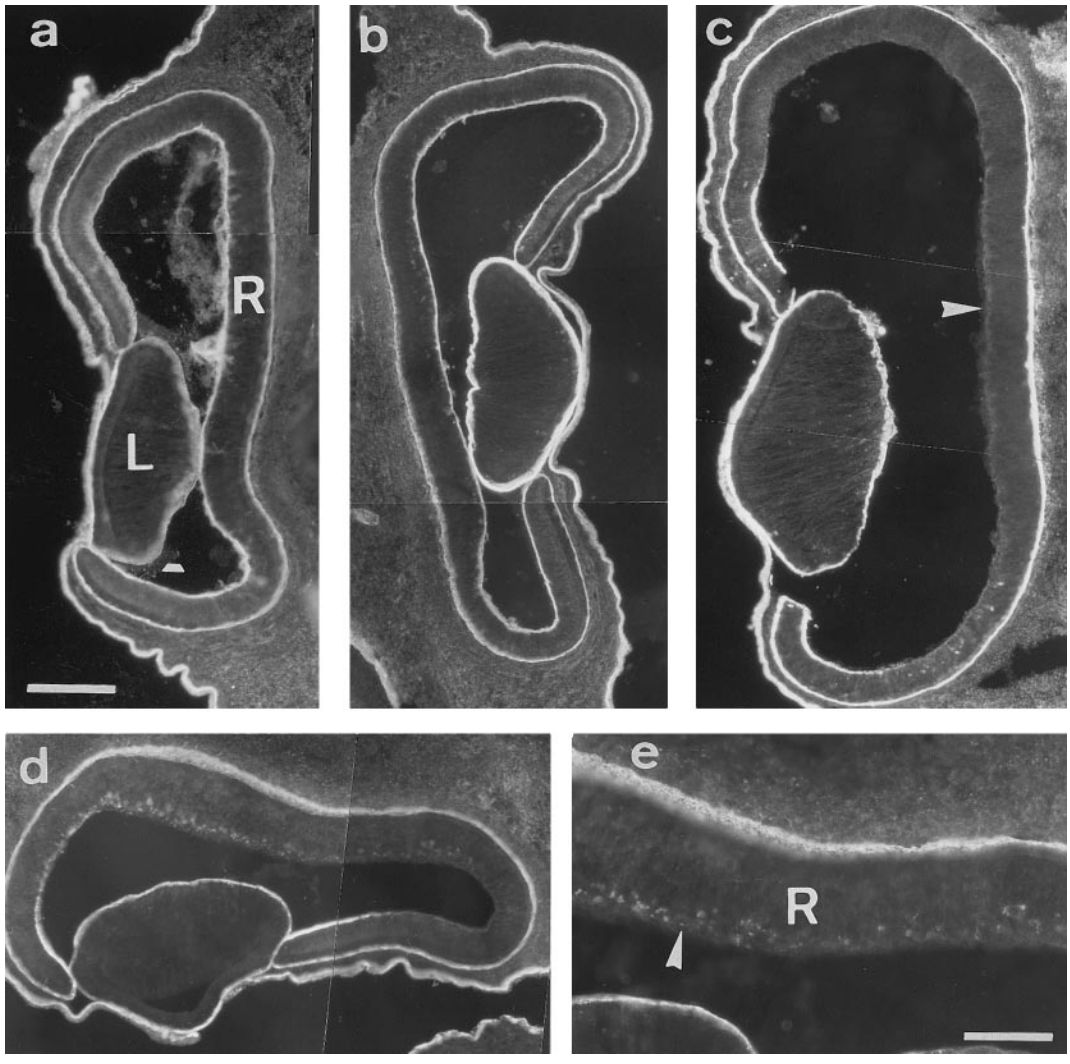


FIG. 8. Cross-sections through E3 chick eyes 8 h after the injection of a mixture of collagenase with Matrigel (a), fetal calf serum (c), or collagen I (d, e). Coinjection of collagenase with Matrigel neutralized the basal-lamina-disruptive activity of the collagenase and resulted in a basal lamina of the injected eye (a) that is indistinguishable from the basal lamina of the contralateral, noninjected eye (b). After coinjection of collagenase with fetal bovine serum (c), or with collagen I (d, e), the retinal basal lamina was no longer detectable (c, arrowhead) or present only in minor fragments (e, arrowhead). L, lens; R, retina. Bar: a–d, 200 μm; e, 100 μm.

regenerated retinal basal lamina, as found by staining of sections with antibodies specific to basal lamina proteins from Matrigel (not shown). Mouse laminin 1 was partially incorporated into the regenerated chick retinal basal lamina (Fig. 9e), but was much less abundant than the chick-derived laminin (Fig. 9f).

Another series of experiments showed that regeneration of the retinal basal lamina could be accomplished any time after its collagenase-induced disruption: a chase with Matrigel 6, 8, 24, or 48 h after collagenase injection resulted 24 h later in regenerated basal laminae in all cases (not shown; $n = 32$).

To determine the time course of basal lamina regeneration, the retinal basal lamina was removed by intraocular injection of collagenase, and the collagenase was chased with Matrigel after a fixed time period (8 h). The status of basal lamina reassembly was then investigated various time intervals after the Matrigel chase by immunocytochemistry on cross-sections through the eyes. Three hours after Matrigel injection, in one of four embryos a fragmented staining of laminin, nidogen, collagen XVIII, and agrin was detectable close to the vitreal surface of the retina indicating that basal lamina reassembly was under way but not complete (not shown). Six hours after Matrigel injection (Fig. 10a; $n = 6$), a new, continuous retinal basal lamina was detectable in all cases, though in two cases, the labeling

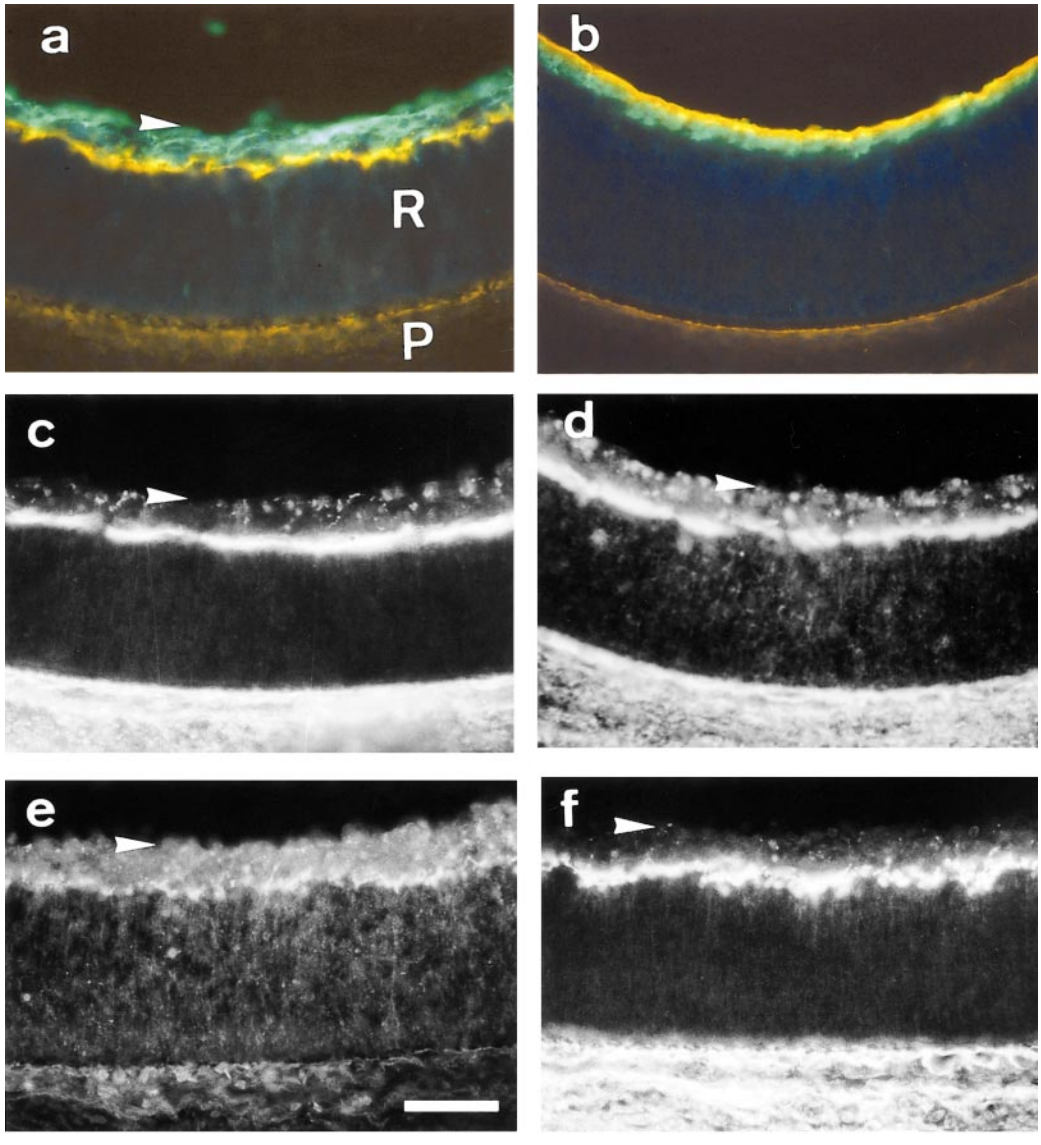
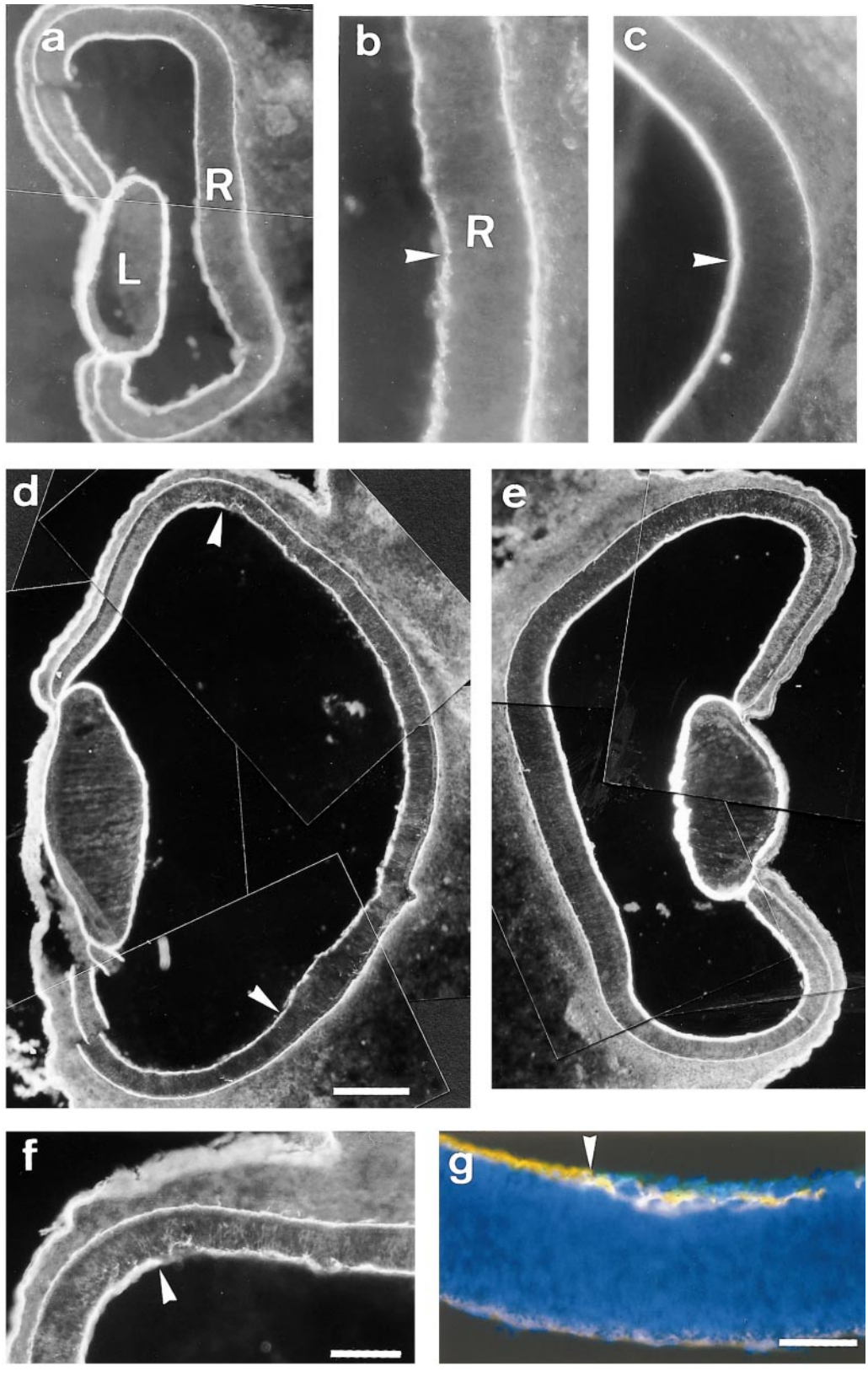


FIG. 9. Basal lamina regeneration in embryonic chick retina after sequential injections of collagenase and Matrigel. The eyes were injected with collagenase at E5, followed by Matrigel injection at E6. Twenty-four hours after the Matrigel injection, cross-sections of the E7 chick retina were double stained with antibodies to laminin (orange) and neurofilament (green, a, b). In eyes injected with collagenase followed by Matrigel, the retinal basal lamina is located underneath the optic fiber layer (a), whereas in a control retina (b), the retinal basal lamina is on top of the optic fiber layer. The displaced location of the regenerate basal lamina is also evident by staining with antibodies to nidogen (c) and collagen XVIII (d). Staining of two adjacent sections with antibodies specific to either mouse laminin or chick laminin show that the laminin of the regenerated retinal basal lamina is predominantly chick-derived (f) rather than from the mouse-derived Matrigel (e). The surface of the retina is indicated by arrowheads. P, pigment epithelium. Bar, 50 μm.

FIG. 10. Time course of basal lamina regeneration. Eight hours after collagenase injection into E3 chick eyes, it was chased by an injection of Matrigel. The embryos were allowed to survive for 6 (a, b) or 12 h (d). The cross-sections through the eyes were stained with antibodies to laminin to reveal the presence of the retinal basal lamina (a–g). A basal lamina has formed within 6 h after Matrigel injection (a, b). The regenerated basal lamina (arrowhead in b) is irregular and the laminin staining is not as strong as in the noninjected contralateral control retina (arrowhead in c). Twelve hours after Matrigel injection, the regenerated basal lamina (d) in the retinal center is only distinguishable from control basal laminae (e) by its irregularities but not by the staining intensity (compare d and e). The basal lamina from the newly developed part of the retina has formed at the vitreal surface of the retinal tissue, whereas the regenerated basal lamina has a location deeper into the retinal tissue. High power views show segments of regenerated and normal basal lamina (f and g). The border of newly developed and regenerated basal lamina is indicated in (d), (f), and (g) by arrowheads. Bar: a, d, and e, 200 μm; b, c, and f, 100 μm; g, 50 μm.



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of the regenerated basal lamina with all basal-lamina-specific antibodies was visibly weaker than that of the contralateral eye (compare Figs. 10b and 10c). Eight to 24 h after Matrigel injection, a new and strongly labeled retinal basal lamina was detected in all cases (Figs. 10d and 10f; n = 36).

Since the retina grows peripherally during embryonic development, the pulse-chase experiments of collagenase followed by Matrigel consistently resulted in eyes with a regenerated basal lamina in the central portion of the retina and a newly synthesized basal lamina in the retinal periphery (n = 24) with a sharp border between the two segments (Figs. 10d, 10f, and 10g).

The ultrastructural status of regenerated retinal basal laminae was investigated by TEM and compared with the ultrastructure of intact retinal basal laminae. High power views showed that 10 h after a Matrigel chase the regenerated basal laminae existed as a thin sheath of extracellular matrix on top of the neuroepithelial endfeet. A basal lamina was never detected on the surface of adjacent axons or ganglion cells (n = 2; Fig. 11a). The neuroepithelial endfeet were identified by their numerous glycogen granules in the cytoplasm and were clearly distinguishable from adjacent ganglion cells and axons (Fig. 11b). During further incubation, the matrix sheath increased in thickness, and 15–20 h after the Matrigel chase, a lamina densa of the regenerated basal lamina could clearly be identified (n = 2; Fig. 11b). Further in development (n = 1; Fig. 11c), the regenerated further increased in thickness, but did not become quite as continuous and compact as an intact basal lamina (Fig. 11d).

To confirm the localization of the regenerated basal lamina along the neuroepithelial endfeet, control retinae and retinae with regenerated basal laminae were double labeled to visualize neuroepithelial cells and the basal lamina at the same time. In control retinae, every neuroepithelial cell extended a thin process both to the scleral and to vitreal surface of the retina, whereby the vitreal endfoot was in contact with the retinal basal lamina (n = 5; Fig. 11e). When the retinal basal lamina was enzymatically removed, the vitreal endfeet were retracted by about 50 μm into the retina whereas the scleral processes remained unchanged (n = 10; Fig. 11f), consistent with earlier findings (Halfter, 1998). When the basal lamina was allowed to regenerate, the new basal lamina formed along the retracted neuroepithelial endfeet (n = 12; Fig. 11g) indicating that the endfeet are the preferred site of basal lamina assembly in the retinal neuroepithelium.

To find out whether the removal or the regeneration of the retinal basal lamina is accompanied with an upregulation or changes in the distribution patterns of extracellular matrix protein mRNAs we performed *in situ* hybridization studies with eyes that had been injected with collagenase to disrupt the retinal basal lamina (n = 4) and with eyes that had been pulse-chased with collagenase and Matrigel to induce basal lamina regeneration (n = 11). In both experimental paradigms, the distribution patterns and the staining densities for collagen IV (Figs. 12a and 12b) and perlecan mRNAs (not shown) of experimental and contralateral control eyes were indistinguishable, strongly suggesting that the removal or the regeneration of the retinal basal lamina is not associated with an upregulation of collagen IV and perlecan expression and is not associated with a change in the expression patterns of the two proteins.

DISCUSSION

Protein Composition of the Retinal Basal Lamina and Origin of Retinal Basal Lamina Proteins in the Developing Eye

Immunocytochemistry and Western blotting showed that the retinal basal lamina consists of laminin, nidogen, agrin, perlecan, and collagen IV and XVIII, whereby the identity and composition of the chick retinal laminin chains remains to be established. In addition to laminin, nidogen, perlecan, and collagen IV, all of which are common to basal laminae, agrin and collagen XVIII are additional constituents of the retinal basal lamina. Agrin and collagen XVIII are both newly introduced basal lamina heparan sulfate proteoglycans (Tsen *et al.*, 1995; Halfter *et al.*, 1998) and may substitute for the much less abundant perlecan, a HSPG that has previously been regarded as the principal HSPG in basal laminae (Noonan *et al.*, 1991; Iozzo and Murdoch, 1996). The fact that agrin is in some tissues more abundant than perlecan has already been shown in the kidney glomerular basal lamina (Groffen *et al.*, 1998).

Collagen IV of the chick retinal basal lamina seemed unique, as the protein was not detectable in the retinal basal lamina by immunocytochemistry of aldehyde-fixed tissue despite the fact that all other basal laminae of the very same sections were brightly stained. Since the abundance of collagen IV in retinal basal lamina could be demonstrated by rotary shadowing and Western blotting, we assumed that the collagen IV network in the retinal basal lamina is uniquely masked by other basal lamina constituents and inaccessible to antibody labeling. This assumption was confirmed by showing that collagen IV was detectable in tissue sections after a protease treatment.

As shown by our *in situ* hybridization studies, almost all basal lamina proteins of the neural retina are not synthesized by the retina, but originate from extraretinal tissues of the eye, such as the ciliary body, the optic disc, and the lens epithelium. The studies also show that the distribution pattern of the mRNA for each basal lamina protein in the eye is unique and that the different basal lamina proteins have different tissue sources. The extraretinal origin of most basal lamina proteins is most likely also true for the earliest stages of eye development for two reasons: *in situ* hybridizations studies for collagen IV, agrin, and collagen XVIII mRNAs from E2.5 onward showed that these proteins

are synthesized by the same cells and tissues at early and later stages of eye development. Further, the developing retina grows by adding new tissue at its periphery, and new neuroepithelium is generated up to E10. A switch in the expression pattern of an mRNA from early to later stages of eye development would have become obvious by a centroperipheral gradient in its mRNA distribution. The only centroperipheral gradient for the expression of a basal lamina protein that was found was that for agrin mRNA, which followed the peripheral-to-central gradient of ganglion cell differentiation in the retina. Since we did not assay eyes beyond E10, it is still possible that in eyes from late stages of embryonic development or from posthatched chickens some of the proteins might also be synthesized by retinal cells as described for laminin and nidogen in the mouse retina (Sarthy and Fu, 1990; Dong *et al.*, 1991).

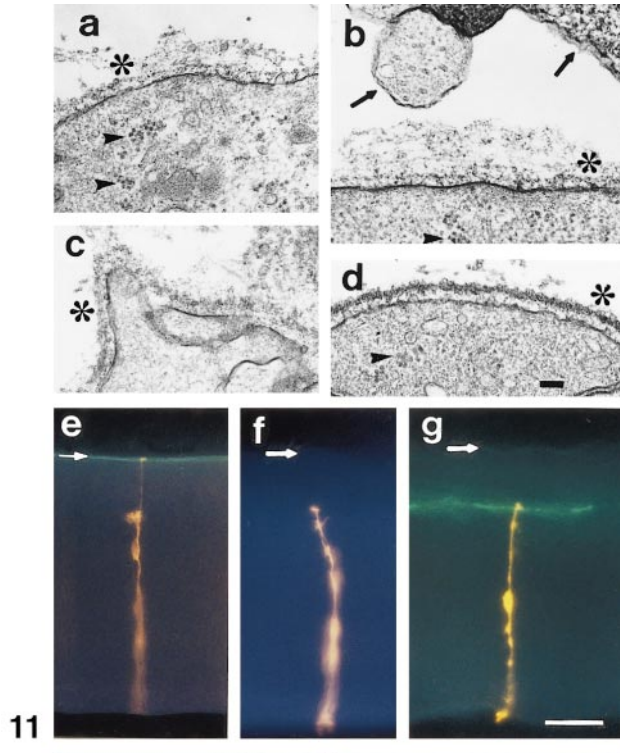
The only basal lamina protein that was found to be synthesized by the neural retina is agrin, with the ganglion cells as the principal producer of the agrin mRNA. Earlier studies have shown that, with the exception of the specialized basal lamina of the neuromuscular junction, agrin is not crucial for basal lamina assembly, since agrin-deficient mice have no obvious abnormalities in the assembly or the structure of basal laminae (Burgess *et al.*, 1999); thus potentially all extracellular matrix proteins that are essential for basal lamina assembly have an extraretinal origin.

Our hybridization data are consistent with previous studies in the mouse eye where all three laminin-1 and collagen IV mRNAs were shown to be synthesized by the lens epithelium and the ciliary body and not by the early embryonic retina. Intravitreal and intraretinal blood vessels, however, are additional sites of synthesis for basal lamina proteins in the mouse eye (Sarthy and Fu, 1990; Dong *et al.*, 1991), and a clear assignment of intraretinal and intravitreal vascular tissue versus extraretinal tissues as the main source of retinal basal lamina proteins has not been possible. The absence of vasculature in the vitreous and the retina in chick, however, shows that lens, ciliary body, and optic disc are able to provide the entire set of proteins necessary to assemble a complete basal lamina at the retinal surface.

Since the site of synthesis and the final tissue location of the basal lamina proteins are separated by the vitreous body, the basal lamina proteins must be secreted from lens, ciliary body, and optic disc into the vitreous body and from there presented to cell surface receptors of the retinal neuroepithelium. Consistent with this notion is that all proteins found in retinal basal lamina were detected in the vitreous body. Further, transplantation experiments showed that the retina is not able to establish a new basal lamina without vitreous body. Thus, the vitreous body represents a storage compartment for basal lamina constituents that allows the continuous and instantaneous assembly of the retinal basal lamina as the surface area of the retina expands more than 50-fold between E2 and E10 (Halfter *et al.*, 1985).

Retinal Basal Lamina Formation in the Developing Eye

The present transplantation data are consistent with the *in situ* hybridization studies, as they show that all proteins necessary to build a new basal lamina do not originate from the retinal transplants but from the host eye with the host vitreous body as a critical component. The role of the retinal neuroepithelium in basal lamina formation is to provide appropriate receptors for the immobilization of one or several basal lamina proteins which then serve as an anchor for other proteins to associate to a complete basal lamina. The receptor proteins for basal lamina nucleation are present on the surface of neuroepithelial tissues from the central nervous system, but not in peripheral nerve tissues and connective tissue. The cells responsible for basal lamina nucleation in CNS neuroepithelium are the neuroepithelial cells, whereby the vitreal endfeet are the specific sites where the basal lamina localizes (Fig. 11). This is most evident in experiments where the basal lamina was allowed to regenerate: the newly formed basal lamina localized exactly where the endfeet of the neuroepithelial cells had been retracted. Surprisingly, when retinal tissue was transplanted with inverse orientation, a new basal lamina was formed over the normally basal-lamina-free scleral side of the retinal neuroepithelium, showing that the proteins of the neuroepithelial cells that nucleate a new basal lamina can also cluster at the scleral side. Candidate receptor proteins for basal lamina nucleation are the members of the integrin family and the laminin-binding dystroglycan. Both types of membrane proteins are expressed by retinal and brain neuroepithelium and both proteins have been shown to have a role in basal lamina assembly (De Curtis *et al.*, 1991; Bloch *et al.*, 1997; Sasaki *et al.*, 1998; Henry and Campbell, 1998). However, unlike the integrins, which are present throughout all retinal cell layers (Cann *et al.*, 1996), dystroglycan is specifically localized to the neuroepithelial endfeet at the vitreal and pial border next to the retinal and pial basal lamina (Blank *et al.*, 1997). Upon removal of the retinal and pial basal lamina with collagenase, the focal dystroglycan staining disappeared from the vitreal surface, and when the basal lamina was allowed to regenerate, brightly stained dystroglycan clusters reappeared exactly where the newly formed basal lamina was located (Halfter, unpublished). Dystroglycan staining also appeared at the scleral side of the retinal neuroepithelium next to the newly forming basal lamina when retinal grafts were transplanted with the scleral side facing the vitreous; thus dystroglycan repolarized to ectopic sites of the neuroepithelium concomitant with the ectopic assembly of a new basal lamina (Halfter, unpublished). The distribution of β1 integrin remained unchanged both during basal lamina removal and during reassembly and did not alter when retinal grafts were transplanted with inverse orientation. Thus, the perfect temporal and spatial match of dystroglycan clustering and retinal basal lamina formation makes dystroglycan, rather than the integrins, a prime



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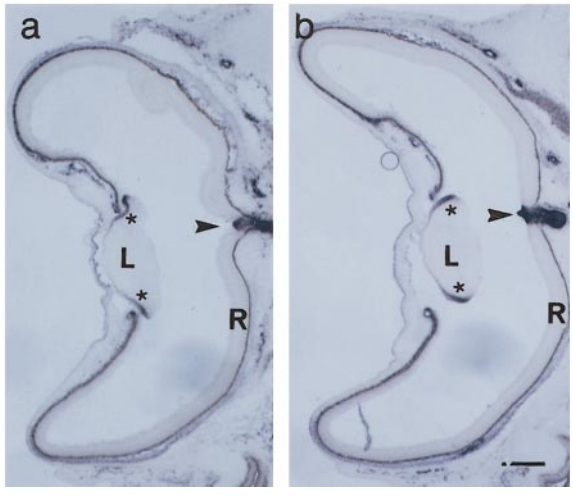

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FIG. 11. Transmission electron micrographs showing the ultrastructure of the regenerated retinal basal lamina after consecutive injections of collagenase and Matrigel. The collagenase was injected into E3 chick eyes followed by a Matrigel chase 10 h later. Ten hours after the Matrigel chase (a), a rudimentary basal lamina has formed on top of the neuroepithelial endfeet (a). The endfeet were identified by their numerous glycogen granules (arrowheads). Twenty hours after the Matrigel chase (b), the regenerated basal lamina has increased in thickness, and a lamina densa is visible. Note that axons (arrows in b) on top of the regenerated basal lamina are not covered by a basal lamina. Thirty hours after Matrigel injection (c), the basal lamina has further increased in thickness, but has still not attained the uniform and even morphology of a control basal lamina (d). Double labeling with DiI (orange) and anti-laminin (green) shows the colocalization of the retinal basal lamina and neuroepithelial endfeet in a normal retina (e) and a retina with a regenerated basal lamina (g). The retraction of the neuroepithelial endfeet after basal lamina disruption is shown in (f). The vitreal surface of the retina is indicated by arrows. Bar: a–d, 50 nm; e–g, 50 μm.

FIG. 12. Light micrographs of cross-sections through E5 chick eyes showing the distribution of α1 collagen IV mRNA in the chick eye after collagenase–Matrigel pulse-chase injections (a) and in an age-matched control eye from another noninjected embryo (b). In the experimental and control eye, the collagen IV mRNA is present is a similar distribution pattern in the lens epithelium (L, asterisks) and the optic disc (arrowheads). The density of staining also suggests that the mRNA is expressed in similar quantities in the experimental and the control eye. Bar, 500 μm.

candidate as a nucleation receptor necessary for basal lamina assembly in the retina and brain.

Our regeneration experiments *in vivo* showed that the formation of a new basal lamina occurs within 6 h in the eye. The fast assembly is surprising as in tissue culture experiments the formation of a new basal lamina from endothelial cells or keratinocytes takes weeks to months (Fleischmajer *et al.*, 1998). The short generation time for basal lamina assembly seen in our experiment, however, is consistent with the instant basal lamina formation in the developing embryo, where retina, brain, skin, vasculature, and internal organs considerably enlarge, but nevertheless are endowed with continuous and gap-free basal laminae. In central nervous system development, the continuous presence of the retinal and pial basal lamina is crucial, as pial and retinal basal laminae are necessary for the anchoring of the neuroepithelial cells to the basal surface of the neuroepithelium and for providing a border to separate the brain from the surrounding mesenchyme. When the retinal or pial basal lamina were experimentally removed, the neuroepithelial cells retracted from their original location, and axonal navigation, intracortical migration, and the control of neuron cell numbers were greatly disturbed. Further, without pial basal lamina, growing axons exited the brain and aberrantly entered the meningeal connective tissue, greatly disturbing the formation of major axonal tracts, such as the visual pathway (Halfter, 1998; Halfter and Schurer, 1998). Thus the continuous presence of a pial and retinal basal lamina is an absolute requirement for the development of the central nervous system, and the developing organisms must provide the means to allow the instantaneous and continuous establishment of this structure as the organism grows. The instantaneous establishment of the basal lamina means that its constituents must be present in excess in a compartment adjacent to the neuroepithelium, which in the eye is the vitreous body. We assume that the meninges have a similar storage function for the establishment of the pial basal lamina in the brain.

In situ hybridization studies of eyes after collagenase injection and collagenase/Matrigel pulse chases strongly

suggest that the expression pattern and the abundance of basal lamina protein messages do not change after basal lamina removal or basal lamina regeneration. For example, perlecan and collagen IV mRNAs were expressed by the very same cells and in similar amounts in the experimental and in the contralateral control eyes, showing that the presence or absence of a retinal basal lamina does not regulate the synthesis of perlecan and collagen IV by the lens and the optic disc.

Our experiments also show a critical role of collagens in basal lamina assembly and maintenance. Intraocular and intraventricular injections of collagenase into the developing chick eye led reliably to the complete disruption of the retinal and pial basal lamina, whereas nonspecific proteases needed much higher concentrations to even cause minor damage to these basal laminae, clearly showing that the stability of the neuroepithelial basal lamina is particularly dependent on the integrity of its collagenous components. It is only after the restoration of the collagen supply in the vitreous that the retinal basal lamina could regenerate, again demonstrating that collagens are crucial for the reassembly of the retinal basal laminae. Obviously, without an intact collagenous network, basal laminae of the retina are instable and fall apart, and without a continuous collagen supply, a new basal lamina cannot form. Our present data are consistent with the notion that a network of collagen IV oligomers provide a scaffold for the other basal lamina proteins to associate with (Yurchenko and Ruben, 1987; Yurchenko, 1994).

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